REMARKS

Claims 83-114 are pending in this application. Claim 83 has been amended. Claims 96-97 and 101 have been canceled. New claims 102-114 have been added herein. No new matter has been added. For the reasons discussed herein, reconsideration is respectfully requested.

Claim Objections

The Examiner has objected to the phrase "a secreted polypeptide" in claim 83, alleging that a polypeptide consisting of the amino acids recited in SEQ IN NO:2 would not constitute a secreted polypeptide because it lacks a signal peptide. Claim 83 has been amended accordingly.

Claim 96-97 and 101 are objected to under 37 C.F.R. 1.75(c) as being of improper dependent form for allegedly failing to further limit the subject matter of the previous claim. As these claims have been cancelled, Applicants respectfully submit that these objections are moot.

Section 112 Rejections

The Examiner has rejected claims 83-97 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Examiner alleges that these claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. As claims 96 and 97 have been canceled, this rejection is moot with respect thereto. These grounds of rejection are traversed with respect to claims 83-95.

The present invention is directed to the important discovery that expression of apolipoprotein E fragments (truncated at the C-terminal region of full length apolipoprotein E) are able to lower total serum cholesterol without inducing hypertriglyceridemia. Prior to the present invention, the beneficial effects of apolipoprotein E in cholesterol homeostasis had been shown, however the therapeutic value of apolipoprotein E in gene therapy approaches was very limited due to the severe hypertriglyceridemia and VLDL accumulation that may be triggered by apolipoprotein E overexpression.

In this regard, four fragments (apoE4-185, apoE4-202, apoE4-229, and apoE4-259) were made and tested in the present invention, and it was shown in all instances that these apolipoprotein E fragments successfully lowered plasma cholesterol levels without causing

hypertriglyceridemia. As taught in the instant Specification, these findings indicate that the amino-terminal residues 1-185 of apolipoprotein E contain all the determinants required for clearance of the lipoprotein remnants which accumulate in the plasma of the apoE-/- mice (Specification, page 30, lines 13-20, and FIGS. 8A, 8B, 16A and 16B). As further discussed in the instant Specification, it was discovered that mice expressing apoE4-185 and apoE4-259 exhibited a reduced rate of VLDL triglyceride production compared to mice expressing full length apolipoprotein E4, indicating that the carboxy terminal region of apolipoprotein E influences the rate of VLDL triglyceride secretion, and that this reduced rate of VLDL triglyceride production may contribute to the inability of the truncated apolipoprotein E mutants to trigger hypertriglyceridemia (Specification, page 36, lines 12-17).

Applicants respectfully point out that description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. See In re Bell, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993), and In re Baird, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994). Moreover, as is the case here, generic claims involving chemical materials provide adequate description of the claimed genus when one skilled in the art can distinguish the genus from others and identify many of the species that the claims encompass. The description need only describe in detail that which is new (Hybritech v. Monoclonal Antibodies, 802 F.2d at 1384, 231 USPQ at 94; Fonar Corp. v. General Electric Co., 107 F.3d at 1549, 41 USPQ2d at 1805.) which, in the present case is, again, the discovery that truncations of full length apolipoprotein E at its C-terminus result in apolipoprotein E fragments which are able to lower plasma cholesterol levels without causing hypertriglyceridemia, and in particular that these characteristics have been shown in fragments having at least residues 1-185 of full length apolipoprotein E. As noted in MPEP §2163, this is equally true whether the claimed invention is directed to a product or a process.

From at least the noted teachings that residues 1-185 of apolipoprotein E contain all the determinants required for clearance of lipoprotein remnants, and that the carboxy terminal region influences triglyceride secretion (and further from the specific working examples that fragments in which residues 186-299, 203-299, 230-299, and 260-299 may be removed from full length apolipoprotein E to produce fragments capable of lowering plasma cholesterol levels without causing hypertriglyceridemia), Applicants respectfully submit that one of skill in the art is

readily capable of making the species falling within the claimed invention and determining, also from the teachings provided in the instant Specification, if such species are capable of lowering plasma cholesterol levels without causing hypertriglyceridemia (in contrast to the Examiner's allegation at page 4 of the Office action that the instant Specification "lacks sufficient guidance" to do so).

For at least the reasons discussed above, Applicants respectfully submit that the instant Specification provides adequate written description for each of the claims, and therefore withdrawal of each of the rejections under Section 112, first paragraph, is appropriate and is respectfully requested.

The Examiner has rejected claim 101 under 35 U.S.C. §112, first paragraph, as not enabled, alleging that the instant Specification does not teach one skilled in the art how to make and use replication-defective adenoviral vectors comprising a nucleic acid encoding amino acids 1-277 of an apoE preprotein of SEQ ID NO:15 in the claimed methods. Claim 101 has been cancelled and generally corresponds to new claim 110, and therefore this rejection will be discussed with respect thereto.

Claim 110 is directed to the expression of apolipoprotein E fragments lacking residues 260-299, which, as noted by the Examiner, is the range of amino acids lacking in each of the working examples (fragments apoE4-185, apoE4-202, apoE4-229, and apoE4-259). As such, fragments lacking this range are clearly taught by the instant Specification. The Examiner alleges, however, that the instant Specification does not provide sufficient guidance for one skilled in the art to modify the residues in this 260-299 range in order to produce species possessing the claimed therapeutic properties. Applicants respectfully disagree.

The Examiner focuses on the alleged unpredictability in the gene therapy art, and the fact that identification of residues within the carboxy terminal region which mediate the hypertriglyceridemic effect was, at the time of the instant filing, the subject of ongoing research. However, Applicants respectfully submit that these arguments are misplaced. Applicants again state that the present invention is directed to the discovery that apolipoprotein E fragments (truncated at the C-terminal region of full length apolipoprotein E) are therapeutically useful for lowering total serum cholesterol without inducing hypertriglyceridemia, solving a significant problem that had existed with the therapeutic use of apolipoprotein E prior to the present

invention. Applicants respectfully submit that it is from this new teaching, namely that apolipoprotein E may be truncated in the C-terminal region to have these noted therapeutic benefits, that enablement is to be determined.

In this regard, the instant Specification includes four working examples of such apolipoprotein E fragments, which show that, in fact, as many as residues 186-299 of full length apolipoprotein E may be removed, while the resulting fragments will still retain the ability to clear cholesterol without increasing triglyceride levels. On the basis of the discoveries in the present invention, it would be expected, of course, that research in this field would continue, for example to determine truncations/modifications that will provide optimal therapeutic benefits, etc. (and in fact such research has continued, as noted by the Examiner). However, with regard to the range set forth in instant claim 110, Applicants respectfully submit that the instant Specification clearly teaches that at least amino acids in the 260-299 range can be removed such that the resulting fragments will still retain the desired therapeutic benefits, and that the those of skill in the art – again, working from the foundation established by the present invention – will be able to modify amino acids within this range, and test the resulting fragments according to the teachings provided in the instant Specification, to determine if they have the desired therapeutic properties.

Applicants note that for a claimed genus, representative examples together with a statement applicable to the genus as a whole will ordinarily be sufficient if one skilled in the art (in view of level of skill, state of the art and the information in the specification) would expect the claimed genus could be used in that manner without undue experimentation, and that an extended period of experimentation may not be undue if the skilled artisan is given sufficient direction or guidance. See MPEP §§2164.02, 2164.06; In re Colianni, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.'" In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing In re Angstadt, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)).

For at least the reasons discussed above, Applicants respectfully submit that each of claims is enabled by the instant Specification and therefore withdrawal of the rejections under Section 112, first paragraph, is appropriate and is respectfully requested.

Section 102 Rejections

The Examiner has rejected claims 83-86, 90-97 and 93-99 as rejected under 35 U.S.C. §102(b) as anticipated by WO 96/14837 ("McClelland) as evidenced by Wetterau (J. Biol. Chem. 263:6240-6248, 1988) and Breslow (J. Biol. Chem. 257:14639-14641, 1982). As claims 96 and 97 have been canceled, these rejections are moot with respect there. With respect to the remaining claims, these grounds of rejection are respectfully traversed.

The Examiner alleges that "McClelland has the same method steps and the same starting materials as the instant broadly claimed methods". (Office action, page 13), and more specifically that the McClelland reference discloses a gene therapy method for the treatment of hypercholesterolemia comprising the intravenous administration of a recombinant replication defective adenoviral vector containing a DNA sequence encoding a human apolipoprotein E3 or a fragment thereof truncated at the C-terminal in a mammalian host. The Examiner alleges that the apolipoprotein E3 C-terminal comprises residues 225-299 according to the Wetterau reference. From this, the Examiner concludes that the McClelland reference anticipates the claimed invention. Applicants respectfully disagree.

Applicants re-state the arguments presented in Applicants' October 1, 2007 Response to these grounds of rejection, pointing out why the McClelland reference is not a proper reference under Section 102 against the instant claims, and will elaborate on those arguments. As discussed, the present invention concerns the discovery that expression of apolipoprotein E fragments truncated at the C-terminal region of full length apolipoprotein E are able to lower total serum cholesterol without inducing hypertriglyceridemia. Specifically, the instant claims are directed to methods for lowering cholesterol in a mammal by administering a replication-defective adenoviral vector comprising a nucleic acid molecule that encodes an apolipoprotein E fragment consisting of the amino acid sequences recited in the claims, wherein the apolipoprotein E fragment, when expressed in a mammal, lowers the total serum cholesterol level without inducing hypertriglyceridemia. Applicants have studied the McClelland reference and respectfully point out that it simply does not disclose such methods, nor does it teach or suggest such methods.

The McClelland reference is directed to treating hypercholesterolemia through the administration of an adenoviral vector (specifically, Av1RE) including DNA encoding full

length apolipoprotein E (Genbank accession #K00396, as noted by the Examiner; and see for example page 11, lines 15-17 of McClelland stating that "the expected full length apoE cDNA, 1,025 bp, was amplified..."). There is simply no disclosure in the McClelland reference of apolipoprotein E fragments having been made, let alone disclosure of fragments having been used therapeutically to lower cholesterol levels without inducing hypertriglyceridemia. With respect to the present invention, McClelland is merely cumulative of prior research directed to the therapeutic use of full length apolipoprotein E (other such references have been discussed, for example, Tsukamoto et al. J. Clin. Invest, 100(1): 107-114, July 1997). As McClelland does not disclose the use of apolipoprotein E fragments to lower cholesterol levels without inducing hypertriglyceridemia, and the Examiner has not shown such disclosure in the McClelland reference, it is not a proper reference under Section 102.

Applicants note that the Examiner relies on the presence of the word "fragments" found throughout McClelland's patent application in making the instant 102 rejections. For example, at page 4, line 5 et seq. of the McClelland reference it states "in accordance with an aspect of the present invention, there is provided an adenoviral vector including a nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof having the biological activity of apolipoprotein E" (emphasis added). As noted by the Examiner, McClelland states that "fragments", as used therein, may result from truncations of apolipoprotein E at its C-terminal end. However, Applicants note that in the same definition, McClelland also states that "fragments" may result from truncations of apoE at its N-terminal end, from amino acid deletions, and/or from mutations. These "fragments" must retain the same biological activity as unmodified apolipoprotein E (McClelland, page 4, lines 19-21).

Applicants point out that each and every recitation of "fragment(s)" in the McClelland reference is in the context of stating that they are "included in" McClelland's invention, but fragments were not actually made nor tested, and therefore were not shown, of course, to lower cholesterol levels without inducing hypertriglyceridemia. Applicants respectfully reminds the Examiner that the disclosure in an allegedly anticipating reference must provide an enabling disclosure of the desired subject matter - mere naming or description of the subject matter, as is the case here, is insufficient. MPEP §2121.01. Applicants also make of record the fact that McClelland's 1995 publication of the research which clearly formed the basis for the filing of WO 96/14837 (Arteriosclerosis, Thrombosis, and Vascular Biology, 1995; 15:479-484; Exhibit

A) makes no mention of "fragments" (and note that the Av1RE vector including full length apolipoprotein E was used in both instances).

Furthermore, Applicants note that the Examiner apparently recognizes these noted deficiencies of McClelland, stating at page 15 of the Office action that "the McClelland et al reference **does not teach** explicitly the use of a fragment of human apolipoprotein E3 that is truncated **at the C-terminus or C-terminal end**...Nor does the McClelland et al reference teach the use of **a fragment of a human apolipoprotein E3 that is truncated within the C-terminal**." (Examiner's emphasis). As such, Applicants respectfully submit that the Examiner apparently recognizes the serious deficiencies of the McClelland reference, but fails to treat them as such. Applicants respectfully request that if the Examiner maintains the Section 102 rejections in view of the McClelland reference, that it be shown where the McClelland reference provides an enabling disclosure of each element of the claimed invention - namely methods involving the use of the recited apolipoprotein E fragments for lowering total serum cholesterol without inducing hypertriglyceridemia, as is necessary for McClelland to be a proper reference under Section 102.

For at least the reasons discussed above, Applicants respectfully submit that the claims are not anticipated by McClelland in view of the other cited references, and therefore withdrawal of each of the rejections under Section 102 is appropriate and is respectfully requested.

Section 103 Rejections

The Examiner has rejected claims 83 and 91-92 under 35 USC §103(a) as obvious over McClelland in view of Wetterau, Breslow, and U.S. Patent No. 6,290,949 ("French"). These grounds of rejection are respectfully traversed.

The Examiner is relying on the McClelland and Wetterau references for the same purposes as in the Section 102 rejection discussed *supra*. Applicants re-state the arguments made in Applicants' October 11, 2007 Response to these grounds of rejection, as well as Applicant's arguments *supra* that McClelland fails to provide an enabling disclosure of apoE "fragments" as used in the present invention, and notes that this deficiency is not remedied by the Wetterau, Breslow and/or French references. Applicants submit that these references, individually or in any combination thereof, fail to disclose each and every element of the claimed invention, as is required to establish a *prima facie* case of obviousness (MPEP §2143).

Specifically, Applicants note that the Examiner has recited the Wetterau reference for its alleged teaching that residues 225-299 make up the C-terminal region of apolipoprotein E, and has recited the French reference for its general disclosure of direct intra-arterial injection of recombinant replication defective adenoviral vectors carrying gene sequences. Applicants note that the Breslow reference has been cited the summary of the above Section 102 rejection and in the summary of the instant 103 rejection, but has not actually been applied in either instance. Nonetheless, Applicants have read the Breslow reference and notes that it merely discloses apolipoprotein E clones, and therefore likewise fails to remedy the noted deficiencies of the McClelland reference.

The Examiner has rejected claims 83-91 and 94-100 under 35 USC §103(a) as obvious over U.S. Patent No. 5,811,243 ("Strittmatter") in view of U.S. Patent No. 6,756,523 ("Kahn") and Breslow. As claims 96 and 97 have been canceled, this rejection is moot with respect thereto. With respect to the remaining claim, these grounds of rejection are respectfully traversed.

Applicants re-state the arguments made in Applicants' October 11, 2007 Response to these grounds of rejection, and elaborates on such arguments herein. Applicants have studied the Strittmatter reference and notes that it is directed to methods for treating Alzheimer's disease – it does not disclose, teach or suggest methods for lowering cholesterol in mammals in need thereof by administering to such mammals replication-defective adenoviral vectors comprising nucleic acid molecules encoding the apolipoprotein E fragments recited in the instant claims, wherein the apolipoprotein E fragments, when expressed in such mammals, lower the total serum cholesterol level without inducing hypertriglyceridemia.

With regard to administering the noted replication-defective adenoviral vectors in the inventive claims to mammals "in need thereof", the Examiner states that "it is noted that lowering cholesterol levels is desirable in any mammal" (Office action, page 24). However, Applicants respectfully points out that no evidence has been cited in support of this assertion, and as such it is merely an assumption by the Examiner which does not provide an appropriate basis for a rejection under Section 103. Additionally, the Strittmatter reference fails to disclose, teach or suggest the use of replication-defective adenoviral vectors of the present invention, but the Examiner alleges that this deficiency is remedied by the Kahn reference for its alleged

teaching of the use of such vectors for the expression of nucleotides in the central nervous system. The Examiner further adds that Breslow had cloned apolipoprotein E4 prior to the present invention (Applicants note that instant inventor Zannis is an author on the Breslow reference). From this, the Examiner alleges that it would have been obvious for one of ordinary skill in the art to modify the methods of Strittmatter by using a replication defective adenoviral vector with apolipoprotein E fragments, as allegedly taught by the other references.

Applicants state that the Examiner has failed to establish a *prima facie* case of obviousness for at least the reasons that the references do not, in combination, recite each element of the claimed invention, nor has the Examiner provided the required reasoning as to why one of skill in the art would have been led to the present invention from the teachings of the cited references.

Specifically, Applicants submit that the cited references together simply do not disclose each and every element of the claimed invention, as required to establish a *prima facie* case of obviousness under Section 103. Specifically, the references do not disclose methods for lowering cholesterol in a mammal in need thereof by administering to such mammals replication-defective adenoviral vectors comprising nucleic acid molecules encoding the apolipoprotein E fragments recited in the instant claims, wherein the apolipoprotein E fragments, when expressed in such mammals, lower the total serum cholesterol level without inducing hypertriglyceridemia. The Examiner's mere assertion that Alzheimer's patients (and, as noted above, "any mammals") are in need of having their cholesterol levels lowered does not establish that this is a fact, and therefore the Examiner has failed to establish that this element of the claims has been satisfied The Examiner has also failed to show, for example, that the administration of apolipoprotein E fragments to such patients lowered their total serum cholesterol level without inducing hypertriglyceridemia - another element of the claimed methods.

Moreover, the Examiner has failed to establish that one skilled in the art would have been led to combine the teachings of the cited references to arrive at the present invention, and has not stated why one skilled in the art would have been so led - asserting only that one skilled in the art would have been motivated to combined the cited references because Kahn "had already taught various advantages for using a recombinant replication defective adenovirus" and because "the human ApoE3 cDNA was already available and cloned in the prior art since 1982". But

Applicants note that there is nothing in the Strittmatter, Kahn or Breslow references discussing deficiencies in Strittmatter's methods of treatment, nor the desirability of improving on them in the manner alleged by the Examiner. The stated reasoning for modifying Strittmatter's methods is only the Examiner's assertion, and is unsupported by either the teachings of the references themselves, or what was otherwise known in the art. (see MPEP §2143 "The key to supporting any rejection under 35 USC 103 is the clear articulation of the reason(s) why the claimed invention would have been obvious", citing KSR International Co. v. Teleflex Inc., 550 U.S. __,___, 82 USPQ2D 1385, 1395-97 (2007); See also MPEP §2142 "...impermissible hindsight must be avoided and the legal conclusion must be reached on the basis of the facts gleaned from the prior art", citing In re Kahn, 441 F.3d 977). For at least the reasons stated above that the cited references fail to disclose each element of the instantly claimed methods, and because the Examiner has not set forth any reasons why the claimed invention would have been obvious in view of the cited references based on the teachings of the references or what was otherwise known in the art, Applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness.

Accordingly, for at least the reasons discussed above, Applicants respectfully submit that the claims are not obvious in view of the cited references and therefore withdrawal of each of the rejections under Section 103 is appropriate and is respectfully requested.

Conclusion

Applicants submit that the claims are in condition for allowance, and a notice to that effect is respectfully requested. Please direct any questions concerning this submission to Applicants' undersigned representative, who can be reached directly at (610) 869-6302.

Respectfully submitted,

Date: 6/30/08

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EXHIBIT A

Arteriosclerosis, Thrombosis, and Vascular Biology

(*Arteriosclerosis, Thrombosis, and Vascular Biology.* 1995;15:479-484.)

Articles

Phenotypic Correction of Hypercholesterolemia in ApoE-Deficient Mice by Adenovirus-Mediated In Vivo Gene Transfer

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Abstract To investigate the potential use of apoE in gene therapy of hyperlipidemias, an adenoviral vector was constructed that contained the human apoE3 cDNA under the control of the RSV promoter (Av1RE). Transduction of HepG2 cells resulted in the overexpression of human apoE secreted into the culture medium. Intravenous injection of $5x10^{11}$ Av1RE vector particles into apoE-deficient mice resulted in expression of human apoE3 in mouse plasma at levels of 1.2±0.4 μg/mL (mean±SEM, n=5) 7 days after injection. Mice injected with the control vector Av1Lacz4 did not express detectable levels of human apoE. Average plasma cholesterol concentrations were reduced approximately eightfold from 737.5±118 mg/dL (mean±SEM, n=6) to 98.2±4.4 mg/dL (mean±SEM, n=5) and were unaffected in the control vector group. Expression of human apoE resulted in a shift in the plasma lipoprotein distribution from primarily VLDL and LDL in the control mice to predominantly HDL in the Av1RE-treated group. Western blot analysis of fast protein liquid chromatography–fractionated mouse plasma showed that the human apoE protein was associated with VLDL, LDL, and HDL. Correction of the hyperlipidemic condition found in the apoE-knockout mouse strain by direct in vivo gene transfer establishes the potential of this approach for treatment of hyperlipidemia caused by apoE deficiency or malfunction in human disease.

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Key Words: atherosclerosis • apolipoprotein E • gene therapy • lipoproteins

Introduction

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ApoE is a component of several plasma lipoproteins, including chylomicrons, VLDL, and HDL. Receptor-mediated catabolism of these lipoprotein particles is mediated through the interaction of apoE with the LDL receptor (LDLR) or with the LDLR-related protein (LRP).[1,2] Injection of exogenous apoE into normal and Watanabe heritable hyperlipidemic (WHHL) rabbits resulted in a decrease in plasma cholesterol concentrations.[3,4] These studies demonstrated that elevation of apoE levels in plasma results in the increased clearance of lipoproteins from the circulation. However, the cholesterol-lowering effect resulting from the intravenous injection of apoE was transient, lasting approximately 20 hours. In apoE-transgenic mice, stable overexpression of apoE resulted in a sustained reduction of plasma cholesterol concentrations and a resistance to dietary elevation of plasma cholesterol concentrations.[5] Kinetic studies of VLDL, LDL, and chylomicron uptake in apoE-transgenic mice showed that overexpression of apoE enhanced the clearance of these lipoproteins from the circulation.[6,7] Taken together, these studies support the hypothesis that apoE overexpression will reduce plasma cholesterol and/or triglyceride concentrations by increasing the clearance of plasma lipoproteins from the circulation.

ApoE-deficient mice are severely hypercholesterolemic, with average plasma cholesterol concentrations of 400 to 800 mg/dL on a regular chow diet.[8,9,10,11] Triglyceride levels in these mice are not severely elevated compared with those in control animals.[8] These mice also develop atherosclerotic lesions at approximately 11 weeks of age with the appearance of foam cells that progress to a more involved, complex lesion consisting of cholesterol clefts and fibrous caps.[10] The severe hypercholesterolemia found in apoE-deficient mice and

the profound effect on lipoprotein metabolism resulting from the deletion of the apoE gene demonstrate that apoE plays a key role in the receptor-mediated clearance of plasma lipoproteins through its interaction with either the LDLR or the LRP.

The generation and characterization of the apoE-deficient mouse strain provide a useful animal model to test various treatments for hyperlipidemias. To investigate the potential of apoE gene delivery to affect hypercholesterolemia, we constructed an adenoviral vector that contains the human apoE3 cDNA. Adenovirus-mediated expression of human apoE in apoE-deficient mice resulted in a complete phenotypic correction of the hypercholesterolemic state.

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Animals

C57BL/6J-Apoe^{mlUnc} apoE-deficient mice were obtained from The Jackson Laboratory. These 10-week-old mice were homozygous for disruption of the apoE gene.[8,12] The mice were fed laboratory mouse chow and allowed to acclimate for approximately 3 weeks before vector administration. Each mouse was treated by tail vein injection with $5x10^{11}$ recombinant adenovirus vector particles, corresponding to approximately $5x10^{9}$ plaque-forming units (pfu) in 400 µL using Hanks' buffered saline solution as diluent.

Lipoprotein and Protein Analysis

Plasma cholesterol concentrations were determined before and after treatment with enzymatic methods (Sigma Chemical Co). Blood collected from either the retro-orbital plexus or the tail vein was immediately transferred to heparinized tubes. Plasma was collected after centrifugation at 7000*g* for 5 minutes. EDTA, Pefabloc, and aprotinin were added to all plasma samples at final concentrations of 2 mmol/L, 1 mmol/L, and 10 µg/mL, respectively.

A 1-µL aliquot of plasma was denatured and applied to a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Novex). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by use of a small transblot apparatus (Biorad) for 30 minutes at 100 V. After the transfer was completed, the PVDF membrane was transiently stained with Ponceau red, and the molecular weight standards were marked directly on the membrane. Molecular weight markers used ranged from 200 to 14 kD (Biorad). The PVDF membrane was blocked in 10 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl, 2 mmol/L EDTA, 0.04% Tween 20, and 5% milk. The blocked membrane was first incubated for 1 hour at room temperature in a 1:3000 dilution of the primary antibody, the anti–human apoE monoclonal clone 3H1 (obtained from Dr Y. Marcel, University of Ottawa Heart Institute). The membrane was developed with a secondary goat anti–mouse IgG1 horseradish peroxidase (HRPO)–conjugated antibody (Southern Biotechnology Associates, Inc) by use of an enhanced chemiluminescence system (Amersham Lifesciences). The membrane was exposed to film for approximately 1 to 10 seconds. Purified human apoE (Calbiochem) was used as a positive control on all Western blot analyses.

Equivalent volumes of plasma from each mouse were pooled per treatment group, and 200 µL was applied to

a Superose 6 gel filtration fast protein liquid chromatography (FPLC) column (Pharmacia). The column was equilibrated in 10 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl, 2 mmol/L EDTA, and 0.02% sodium azide at a flow rate of 0.35 mL/min, and 0.5-mL fractions were collected. Cholesterol was determined on 100 μL of each fraction by enzymatic methods. Purified human VLDL and HDL (Calbiochem) were used to calibrate the column.

Cloning of the ApoE cDNA

The apoE cDNA was constructed by use of gene overlap extension polymerase chain reaction (PCR) methods[13] with Pfu DNA polymerase (Stratagene) in the presence of 10% dimethyl sulfoxide. The 5' end of the apoE cDNA, nucleotides -39 to 292, was generated with liver cDNA (Clonetech) as the template with the following primers: P1, 5'-ACTCAGCCCCAGCGGAGGTGAAGGACGTCCTTCCCCAGGAGCCG-3'; P2, 5'-TTCCTCCAGTTCCGATTTGTAGGCCTTCAACTCCTTCATGGTCTCGTC-3'. The primer P1 was designed to start at the major transcription initiation site in exon 1.[14] The PCR was carried out with the following conditions: 95°C for 10 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 1 minute, 72°C for 2 minutes, and finally a 72°C extension for 10 minutes. The 3' end of the apoE cDNA was amplified from the cloned apoE fragment[15] EB4 (obtained from Dr Steve Humphries) with the primers P3 (5'-GCCTACAAATCGGAACTGGAGGAA-3') and P4 (5'-AGGCTTCGGCGTTCAGTGATTGT-3') to produce a 696–base pair (bp) fragment. The 5' and 3' PCR apoE fragments were gel-purified. The PCR was performed with equal volumes of the melted fragments and the end primers P1 and P4. The expected full-length apoE cDNA, 1025 bp, was amplified and ligated directly into the pCRII vector (Invitrogen). Several clones were screened by restriction enzyme analysis and sequenced. A clone matching the expected sequence (GenBank accession No. K00396) was selected.

Recombinant Adenoviruses

A recombinant adenovirus vector containing the human apoE3 cDNA was constructed by use of the vector system described previously.[16] The apoE3 cDNA was placed downstream of the RSV promoter in an adenoviral backbone deleted for E1a, most of the E1b region, and the E3 region. The 293 cells were cotransfected with *Kpn* I–linearized pAvS6E and the large *Cla* I fragment of Ad5dl327. This transfection produced the recombinant adenovirus containing the apoE3 cDNA and was called Av1RE. Recombinant adenoviral plaques were identified with PCR to detect the apoE3 cDNA and were expanded in 293 cells. The adenovirus titers (particles per milliliter) were determined spectrophotometrically[17] [18] and compared with the biological titer (pfu per milliliter). The ratio of total particles to infectious particles (particles per pfu) was usually 100 or less. Fig 1 schematically shows maps of Av1LacZ4 and Av1RE vectors.

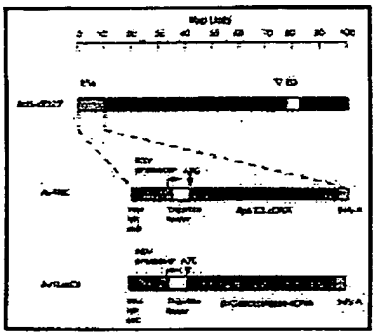

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Figure 1. Schematic representation of the adenoviral vectors. The human apoE3 cDNA or the ß-galactosidase cDNA was placed downstream of the RSV promoter and the adenoviral tripartite leader. The cDNA sequences were transferred into the Ad5dl327 genome by homologous recombination. Av1RE and Av1LacZ4 are the adenoviral vectors containing the human apoE3 cDNA and the ß-galactosidase cDNA, respectively.

Cell Culture

HepG2 cells were cultured in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS). Transductions were carried out in EMEM containing 2% FBS, 100 U/mL penicillin, and 10 µg/mL streptomycin when the cells had reached approximately 90% confluency. The adenoviral vector was diluted in 0.5 mL of the transduction medium and was placed on the cell monolayer for 1.5 hours at 37°C. The medium was removed, and 1 mL of fresh transduction medium was then added. After 24 hours, the medium was collected, and Western blot analysis was carried out on a 10-µL aliquot.

Human ApoE Enzyme-Linked Immunosorbent Assay

Human apoE in mouse plasma was measured with a sandwich-type enzyme-linked immunosorbent assay (ELISA) with a mouse monoclonal, 9-H8, as the capture antibody and a goat polyclonal as the detecting antibody. Values were determined by use of a standard curve obtained by the inclusion of varying amounts of purified recombinant human apoE3 (Calbiochem) into plasma from apoE-deficient mice. Microtiter plates (Immulon 4, Dynatech) were coated with the 9-H8 antibody (5 µg protein/mL, Cappel) in 100 mmol/L sodium bicarbonate, pH 9.6, overnight at room temperature. The monoclonal antibody solution was removed, and the plate was washed five times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Unless otherwise noted, all subsequent incubations were carried out for 1 hour at room temperature. The nonspecific protein binding sites in each well were blocked with PBS containing 10% milk. The blocking buffer was removed, and the plates were washed as described previously. The standards and samples were diluted in PBS containing 2% bovine serum albumin (BSA) and allowed to incubate at 4°C. A 100-µL aliquot of each sample or standard dilution was placed in individual wells and allowed to incubate. The plates were washed as described and were then incubated with 100 µL of a 1:4000 dilution of the secondary anti–human apoE goat polyclonal antibody (Calbiochem) in PBS containing 2% BSA. After washing, the plates were incubated with 100 µL of a 1:3000 dilution of the tertiary swine anti–goat IgG-HRPO polyclonal antibody (Caltag). The reaction was developed with 100 µL of 0.2 g/L 3,3',5,5'-tetramethylbenzidine and 0.01% H_2O_2 and was stopped by the addition of 100 µL of 1 mol/L phosphoric acid. The absorbance at 450 nmol/L was measured, with 405 nm as the reference. Human plasma samples were used as controls in each assay, and reproducible values within the reported range of 3 to 5 mg/dL[19] were obtained.

Results

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Adenoviral Expression of Human ApoE In Vitro

An adenoviral vector (Av1RE) containing the human apoE3 cDNA was constructed (Fig 1⊞). HepG2 cells were transduced with the Av1RE vector at multiplicities of infection of 10 or 100 or a control vector, Av1Lacz4, containing a nuclear targeted ß-galactosidase cDNA. The culture medium was analyzed by Western blot analysis with an anti–human apoE monoclonal antibody, 3H1 (Fig 2⊞). Untransduced HepG2 cells[20] and Av1LacZ4-transduced HepG2 cells secreted a low level of apoE that was detected in the culture medium (Fig 2⊞, lanes 3 and 4). However, Av1RE-transduced HepG2 cells secreted substantially higher levels of apoE, indicating that the vector directs the overproduction of apoE in vitro. Vector-derived human apoE had an identical *Mr* of 34 000 compared with both purified human apoE (Fig 2⊞, lanes 1 and 2) and the endogenous protein (Fig 2⊞, lanes 3 and 4)

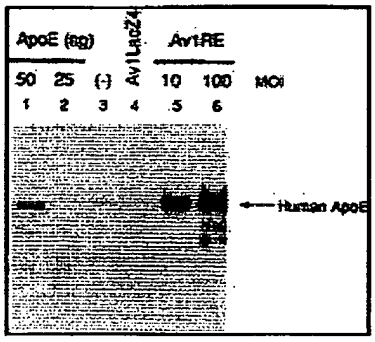

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Figure 2. Western blot shows in vitro expression of human apoE3 from transduced HepG2 cells. HepG2 cells were transduced with either Av1LacZ4 (lane 4) or Av1RE (lanes 5 and 6) at a multiplicity of infection (MOI) of 10 or 100 and compared with untransduced HepG2 cells (lane 3). After 24 hours, the medium was collected and analyzed for human apoE by Western analysis with the anti–human apoE monoclonal 3H1. Purified human apoE (25 or 50 ng) was used as a positive control (lanes 1 and 2).

Adenoviral Expression of Human ApoE In Vivo

To determine whether the human apoE produced by the Av1RE vector could influence cholesterol metabolism in vivo, $5x10^{11}$ viral particles ($5x10^{9}$ pfu) of the Av1RE or Av1LacZ4 vector were administered to apoE-deficient mice by tail vein injection. We and others previously demonstrated that intravenous injection of adenoviral vectors results in preferential and efficient transduction of liver hepatocytes.[16 21 22 23] At 7 days after injection, blood was obtained from the tail vein, and plasma was analyzed by Western blot analysis (Fig 3⊞). Human apoE was detected in the plasma of the Av1RE-treated mice and was not present in the plasma of the Av1LacZ4 vector control group. Human apoE concentrations in mouse plasma were quantified by ELISA. The average apoE concentration in mouse plasma 7 days after vector administration was 1.2±0.4 µg/mL (mean±SEM, n=5), which is approximately 4% of normal human apoE levels.[19]

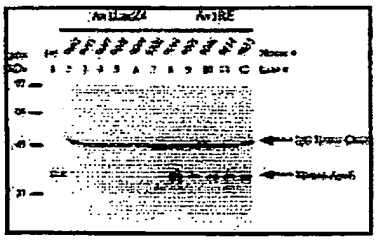

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Figure 3. Western blot shows in vivo expression of human apoE3 in apoE-deficient mice. Plasma from Av1LacZ4-treated (lanes 2 through 7) and Av1RE-treated (lanes 8 through 12) mice was collected. A 1-μL aliquot of plasma was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western analysis. Purified human apoE (100 ng) was used as a positive control (lane 1). The membrane was developed with the anti–human apoE monoclonal 3H1 and goat anti–mouse IgG1–horseradish peroxidase. Arrows show the positions of the human apoE and mouse IgG heavy chain. The individual mouse identification numbers are listed above each lane. Molecular weight (MW) standards are indicated.

Consistent with previous data, the mean plasma cholesterol value in the apoE-deficient mice before vector treatment was 737.5±118 mg/dL (mean±SEM, n=6).[8 9 11 24] One week after administration of the Av1RE vector, plasma cholesterol levels had declined eightfold to a mean of 98.2±4.4 mg/dL (mean±SEM, n=5) (Fig 4), which is equivalent to levels found in normal C57BL6 mice fed a chow diet.[5 8 25] Mice that received the control Av1LacZ4 vector had similar levels of plasma cholesterol before and after treatment, indicating that the reduction in plasma cholesterol concentrations was due to the expression of human apoE.

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Figure 4. Bar graph shows plasma cholesterol concentrations in adenovirus-treated apoE-deficient mice. The average total plasma cholesterol (TPC) concentrations were determined enzymatically on plasma samples obtained before and 7 days after vector administration for the Av1LacZ4-treated (open bars; mean±SEM; n=6) and Av1RE-treated (striped bars; mean±SEM; n=6 pretreatment and n=5 posttreatment) groups. A paired *t* test was carried out on the average TPC values before and after treatment. **P*<.005.

Fig 5 shows the plasma lipoprotein distributions of the Av1RE- and Av1LacZ4-treated mice 7 days after vector administration. Pooled plasma from each treatment group was fractionated with a Superose 6 gel filtration column, and the cholesterol content was measured in each fraction across the elution profile. As expected for untreated apoE-deficient mice, the lipoprotein elution profile of the Av1LacZ4-treated group showed that the majority of the cholesterol eluted in the VLDL-LDL region.[8 9] In contrast, the lipoprotein distribution of the Av1RE-treated animals was shifted so that the cholesterol found in the VLDL-LDL region was reduced and HDL was the primary cholesterol-containing lipoprotein.

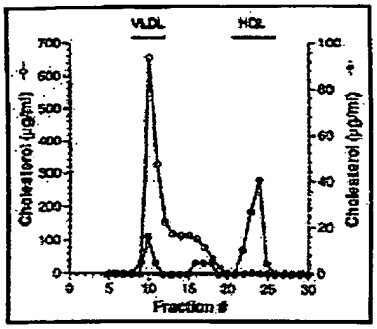

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Figure 5. Line graph shows plasma lipoprotein distributions of adenovirus-treated apoE-deficient mice. Pooled plasma from the Av1LacZ4-treated group (○, n=6) or the Av1RE-treated group (●, n=5) was fractionated with a Superose 6 gel filtration column, and the cholesterol content was determined on individual fractions across the elution profile. The cholesterol concentration (in micrograms per milliliter) was plotted for each fraction collected (note the different *y* axes for each treatment group). The elution positions of purified human VLDL and HDL are indicated.

The plasma lipoprotein distribution of human apoE in the Av1RE-treated mice 7 days after vector administration was confirmed by Western blot analysis of the fractionated plasma samples (Fig 6). The majority of the human apoE was associated with the VLDL-LDL fraction, although a smaller proportion of apoE was detected in the HDL fraction. This result was similar to the plasma lipoprotein distribution of endogenous apoE from normal C57BL6 mice fed an atherogenic diet (data not shown).[26]

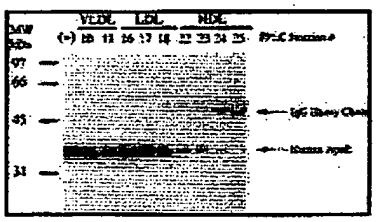

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Figure 6. Western blot shows human apoE distribution among mouse plasma lipoproteins. The Av1RE plasma fast protein liquid chromatography (FPLC) fractions containing cholesterol were analyzed for the presence of human apoE. A 25-µL aliquot of each fraction was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western analysis as described in Fig 3. The FPLC fraction numbers are listed above each lane and correspond to the fractions shown in Fig 5. Purified human apoE (100 ng) was used as a positive control (+). Molecular weight (MW) standards are indicated.

The reduction in total plasma cholesterol levels in the Av1RE-treated apoE-deficient mice persisted for at least 21 days after administration of the adenoviral vector (Fig 7A). The mean plasma cholesterol concentrations in the Av1RE-treated apoE-deficient mice were 98.2±4.4, 215±61.4, and 161.5±26.3 mg/dL (mean±SEM, n=5) at 7, 14, and 21 days after vector treatment, respectively. At 35 days after vector administration, the plasma cholesterol concentrations in the Av1RE-treated group increased to approximately 550 mg/dL, but this was still lower than that seen in the control vector group. A significant change in the plasma cholesterol concentrations was not observed over the course of the study in the Av1LacZ4-treated control mice.

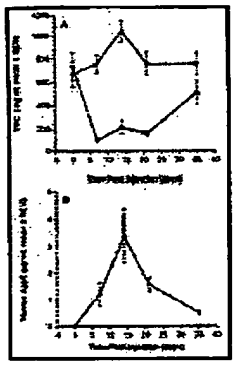

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Figure 7. Line graphs show total plasma cholesterol (TPC) and plasma human apoE3 concentrations after adenoviral vector administration. Plasma was obtained from each mouse at the indicated times. Plasma cholesterol concentrations were determined enzymatically, and human apoE3 concentrations were determined by enzyme-linked immunosorbent assay. TPC concentrations (milligrams per deciliter, mean±SEM) (A) or human apoE concentrations (micrograms per milliliter, mean±SEM) (B) were plotted as a function of time after injection for the Av1LacZ4-treated (○, n=6) and Av1RE-treated (•, n=4 to 5) groups.

Expression of human apoE in mouse plasma persisted for at least 35 days after administration of the adenoviral vector (Fig 7B). The concentration of human apoE varied over the course of the study, with the highest level of 3.4±0.9 μg/mL (mean±SEM, n=4) found at 14 days. The increase in plasma cholesterol concentrations 35 days after injection correlated with a decline in the plasma human apoE concentrations to a level of 0.5±0.1 μg/mL (mean±SEM, n=5) (Fig 7B).

Discussion

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Adenovirus-mediated expression of human apoE in hypercholesterolemic apoE-deficient mice resulted in a transient phenotypic reversion of the hypercholesterolemic state normally found in this mouse strain. The levels of human apoE expression were sufficient to produce a significant lowering of plasma cholesterol concentrations that persisted for at least 21 days after vector administration. Correction of the hypercholesterolemic condition in the apoE-deficient mouse model by direct in vivo gene transfer establishes the potential of this approach for treatment of hyperlipidemias resulting from apoE deficiency[27] or malfunction.[28]

An inverse relation between plasma apoE concentrations and plasma cholesterol levels was reported previously in transgenic mice expressing the rat and human apoE genes.[5,29] These studies demonstrated that a plasma apoE concentration of approximately 90 to 100 μg/mL was needed before a decline in cholesterol levels was found. The current study showed that a human apoE concentration of 1 to 4 μg/mL was sufficient to lower plasma cholesterol levels to the normal range in apoE-deficient mice. Taken together, these studies suggest that in normal animals, substantial overexpression of apoE above the endogenous apoE levels may be required to elicit an effect on plasma lipid levels. However, the severe hypercholesterolemia resulting from gene knockout is affected by relatively modest apoE levels. It will be of interest to test the Av1RE vector in various hyperlipidemic animal models such as the LDLR-deficient mice, WHHL rabbits, apoCIII-transgenic mice, or apoE2-transgenic mice to assess whether apoE gene therapy might be of general benefit in human

disease. In addition to the treatment of hyperlipidemias, the Av1RE vector also could be used to assess the effects of human apoE3 gene therapy on the development of Alzheimer's disease.

The decrease in plasma cholesterol levels observed in the Av1RE-treated mice was accompanied by changes in the plasma lipoprotein distribution (Fig 5). The presence of human apoE in the plasma of the apoE-deficient mice produced a decrease in VLDL and LDL cholesterol and an increase in HDL cholesterol. ApoE-deficient mice have both intestine and liver-derived remnant lipoprotein particles that accumulate in plasma and result in elevated plasma cholesterol concentrations.[8,9] These remnant particles are normally cleared from the circulation through the interaction of apoE with the LDLR, LRP, or both.[2,24] Previous studies showed that the elevation of apoE levels can enhance the clearance of VLDL, LDL, and chylomicrons from the circulation.[6,7] The reduction of plasma cholesterol concentrations and changes in the plasma lipoprotein distribution were presumably the result of the association of the human apoE protein with both apoB48- and apoB100-remnant lipoprotein particles (Fig 6), thereby increasing their rate of removal from the circulation. We have used adenovirus-mediated gene delivery to deliver a functional human apoE cDNA to correct the genetic deficiency found in the apoE-deficient mouse model. The reduction of plasma cholesterol concentrations and changes in the plasma lipoprotein distribution presumably resulted when the human apoE associated with the apoB48- and apoB100-remnant lipoprotein particles, increasing their removal from the circulation. The present study demonstrates that the phenotypic correction of the apoE-deficient mouse can be achieved by transient delivery of the apoE3 gene by use of an adenoviral vector. This approach has also been used to produce a transient phenotypic correction of LDLR-deficient mice and WHHL rabbits with adenovirus-mediated expression of the human LDLR.[30,31]

Normal C57BL6 mice on a chow diet have average plasma cholesterol concentrations that range from 40 to 100 mg/dL.[5,8,25] The Av1RE-treated apoE-deficient mice had plasma cholesterol concentrations similar to those of normal mice for at least 21 days after vector administration (Fig 7A). However, the cholesterol-lowering effect was transient, and the elevation of plasma cholesterol concentrations 35 days after vector administration was correlated with a decline in human apoE concentrations (Fig 7). The decline in plasma human apoE concentrations probably resulted from the loss of the vector DNA from the liver, as found in previous studies.[16,32] However, an immune response to the human apoE3 protein cannot be ruled out at this point as an explanation of the decreased plasma concentrations.

Atherosclerosis develops readily in the apoE-deficient mouse model.[10,11] Evaluation of the effects of apoE gene therapy on the development of atherosclerosis in the apoE-deficient mouse will most likely require a more sustained and persistent gene expression. The current vector, Av1RE, resulted in expression of apoE for at least 21 days, with subsequent cholesterol lowering over that time. However, 21 days may not be long enough to influence the amount and extent of atherosclerosis in these animals. As Yang et al[32] described, the current hypothesis to explain the decline in transgene expression over time suggests that a low level of viral gene expression occurs and invokes a cellular immune response against the adenovirus-infected cells. To achieve persistent transgene expression, current efforts are focused on vector modifications designed to reduce functional expression of adenoviral backbone sequences.[33] With persistent expression of apoE, the evaluation of apoE gene therapy on the development of atherosclerosis can be further studied.

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